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Action spectrum for the induction of hyphal branches of an arbuscular mycorrhizal fungus: exposure sites *versus* branching sites†

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The first action spectrum for a photo-induced response of an arbuscular mycorrhizal fungus is reported. At low light intensity, the responsive wavelengths for light-induced hyphal branching of the primary germ tube of *Gigaspora gigantea* were determined to be in the blue to UV-A range. The action spectrum showed the greatest stimulation of branching occurred around 390 nm although a shoulder was observed between 360–370 nm. A second major peak of light-induced branching occurred at 430 nm. The exposure of specific areas of the germ tube to high intensity blue light for a short period led to several interesting observations. By exposing 2 mm segments (0–2, 2–4, 4–6, etc.) or 3 mm segments away from the tip, it was determined that photoinduction of hyphal branches could occur anywhere along the axis of a growing germ tube except in the apical 2 mm. When 3 mm segments were exposed at greater distances from the tip (6–9, 9–12, and up to 33–36 mm), branches frequently formed in areas not directly exposed to light. The branches were usually in clusters which were spaced approximately 3 or 6 mm apart. Since light scattering could be ruled out, these results indicated that the exposure sites and sites of hyphal branching did not necessarily coincide and suggested the probable involvement of a second messenger during this blue light-induced event.

INTRODUCTION

Although fungi are not able to use light for photosynthetic processes, radiation does play a role in the biochemical and morphological responses of slime moulds (Corrochano & Cerda-Olmedo 1991) and other soil borne fungi (Gressel & Rau 1983). Specifically, the UV-A to blue region of the spectrum (340–500 nm) induces various morphogenetic responses in fungi such as sporulation, carotenogenesis, inhibition and stimulation of conidiogenesis, inhibition of spore germination, and aggregation of fungi (Gressel & Rau 1983, Corrochano & Cerda-Olmedo 1991, Galland 1992, Horwitz & Berrocal 1997).

We have been working with arbuscular mycorrhizal (AM) fungi, which are members of the *Glomeromycota* (Schüßler, Schwarzott & Walker 2001). They are unusual in that they are coenocytic (multinucleate), mutualistic, obligate symbionts which infect about 80% of all land plants. After spore germination, the first interaction between the AM fungus and host plant is the increase in growth (Bécard & Piché 1989) and proliferation of hyphal branches in response to compounds exuded by the host root (Giovannetti *et al.* 1993). The branching increases the probability that a hyphal tip will come in contact with a site in an epidermal groove of the root and start the infection process. We are currently assessing other environmental factors which also stimulate hyphal branching. Although little information is available on photo-induced hyphal branching of fungi in general (Raudaskoski & Viitanen 1982, Grinberg & Heath 1997, Lauter *et al.* 1998), even less is known about this in AM fungi (Nagahashi, Douds & Buee 2000). We have determined the first action spectrum for light-induced hyphal branching of any fungus, and have looked at blue light-induced hyphal branching of an AM fungus in greater detail.

MATERIALS AND METHODS

Fungal spores

Zygospores of *Gigaspora gigantea* were produced in pot cultures in a greenhouse with *Paspalum notatum* as a host. Spores were isolated from pot culture media via

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wet sieving and centrifugation (Gerdemann & Nicolson 1963, Jenkins 1964) and surface-sterilized with chloramine T, streptomycin, and gentamicin (Bécard & Fortin 1988). Aseptic spores were germinated on M medium solidified with 0.4% gellan (w/v) in square Petri plates (Bécard & Fortin 1988) and grown for at least three days at 32 °C in 2% CO_2. The primary germ tube of *G. gigantea* is negatively geotropic and Petri plates containing the germinated spores were always illuminated or incubated in an upright position (Petri plate on edge) so that the germ tubes were allowed to grow straight up. Voucher material is preserved in green house pot cultures in USDA ARS (Wyndmoor).

Dose dependence and action spectrum

Dose-dependent responses to various wavelengths were used to determine the action spectrum for hyphal branching. An Oriel (Stratford, CT) model 77205 monochromator fitted with a tungsten-halogen lamp was used as the light source and a very sensitive IL 1700 Research Radiometer with an SED 033 detector (International Light, Newburyport, MA) was used to determine light intensity. The radiometer had calibration factors determined at 10 nm increments for a tungsten-halogen lamp and this permitted a direct readout in $\mu E\ s^{-1}\ cm^{-2}$ (converted to $\mu moles\ s^{-1}\ m^{-2}$) between 340–450 nm. At wavelengths between 450–500 nm, the readouts were the same as those achieved with a Li-Cor (LI-189) Quantum/Radiometer/Photometer (Lincoln, NE) with an LI-190SA quantum sensor. The Li-Cor system was used for all wavelengths above 500 nm. During the exposure time, especially with high light intensities, one fan was used to blow air over the light source and a second fan was used to blow directly on the Petri plate to minimize any heating effects.

An aluminum foil patch with a slit (1.5 mm wide and 12 mm high) was taped to the Petri plate prior to exposing the germinated spore to light. The slit was used to center the negatively geotropic germ tube (an 8 mm apical segment that had no previous lateral hyphal branch). The extra 4 mm of the slit length was to allow for the growing tip to be continuously exposed during long exposures. The light emitted from the monochromator came from a narrow slit which was then centered on the aluminum foil slit. The germ tubes were illuminated at various wavelengths with exposure times varying between 2–24 h. After the exposure time, the plates were transferred to a CO_2 (2%) incubator at 32 ° in the dark for an additional 24 h to optimize fungal growth (Bécard & Piché 1989). Branches then were counted using a stereomicroscope at 20 × to 50 × magnification. Data were analyzed by plotting hyphal branches induced (dependent variable) *versus* the total dosage of light received (independent variable). Regression equations were calculated and plotted using GraphPad Prism software (GraphPad software, San Diego, CA). Each point on the graphs represents the average of 2–4 replicates at each dosage with four or more different dosages used for each wavelength.

Short-term exposure to high intensity filtered blue light

To expose the germ tube at various distances from the apex, long-term exposures could not be used because of the continuous growth of the apical tip. The germ tubes can grow between 5–15 mm in 22 h depending on the environmental conditions, so short-term (5–30 min) high intensity blue light was necessary to minimize hyphal tip growth during the irradiation period. The monochromator system could not be used, so filtered blue light was employed. An MKII fiber optic light unit (EPOI, Garden City, NY) with a tungsten-halogen lamp was used and blue light intensity was determined as stated above with the radiometer calibrated for 390 nm. To reduce harmful UV rays below 340 nm, a long pass UV 345 filter in conjunction with a 4 mm thick blue filter was used to provide UV-A to blue light. The light transmitted through the filtering system directly overlapped the significant wavelengths of the action spectrum with maximum transmission at 390 nm.

For the time course and fluence rate response curves, the fibre optic probe was placed 1–2.5 cm from the filters which were taped over the aluminum foil slit (12 × 1.5 mm) on the Petri plate. Fans were placed near the light source and the Petri plate to minimize heating and the two filters also acted as a heat shield to prevent the plate from warming up during the exposure time. To expose specific apical tip lengths or specific segments of the germ tube, aluminum foil slits of various lengths were used. With this technique, various segments of germ tubes could be exposed and the exposed segments could be varied in length and distance from the growing tip. The number of replications for each experiment is given in the text. Controls were kept in the dark at room temperature (23 °) or light was allowed to shine on the Petri plate which was completely covered in foil (no slit) for 10, 20, and 30 min before transferring to the CO_2 incubator for 16 h. After this incubation period, controls and experimentals were viewed under a stereomicroscope and branches were either counted directly or tracings of the branching pattern were made right on the Petri plate.

RESULTS

Action spectrum for light-induced hyphal branching

Any hypha from a germinated spore of *Gigaspora gigantea* could be induced to branch with light (Nagahashi *et al*. 2000) however, the primary germ tube was chosen for this study because of its mode of growth. Our initial experiments indicated that light between 450–700 nm at intensities at or less than $3\ \mu m\ s^{-1}\ m^{-2}$ did not induce branching even after 24 h of exposure. A wavelength dependent response between 340–450 nm using a constant intensity of

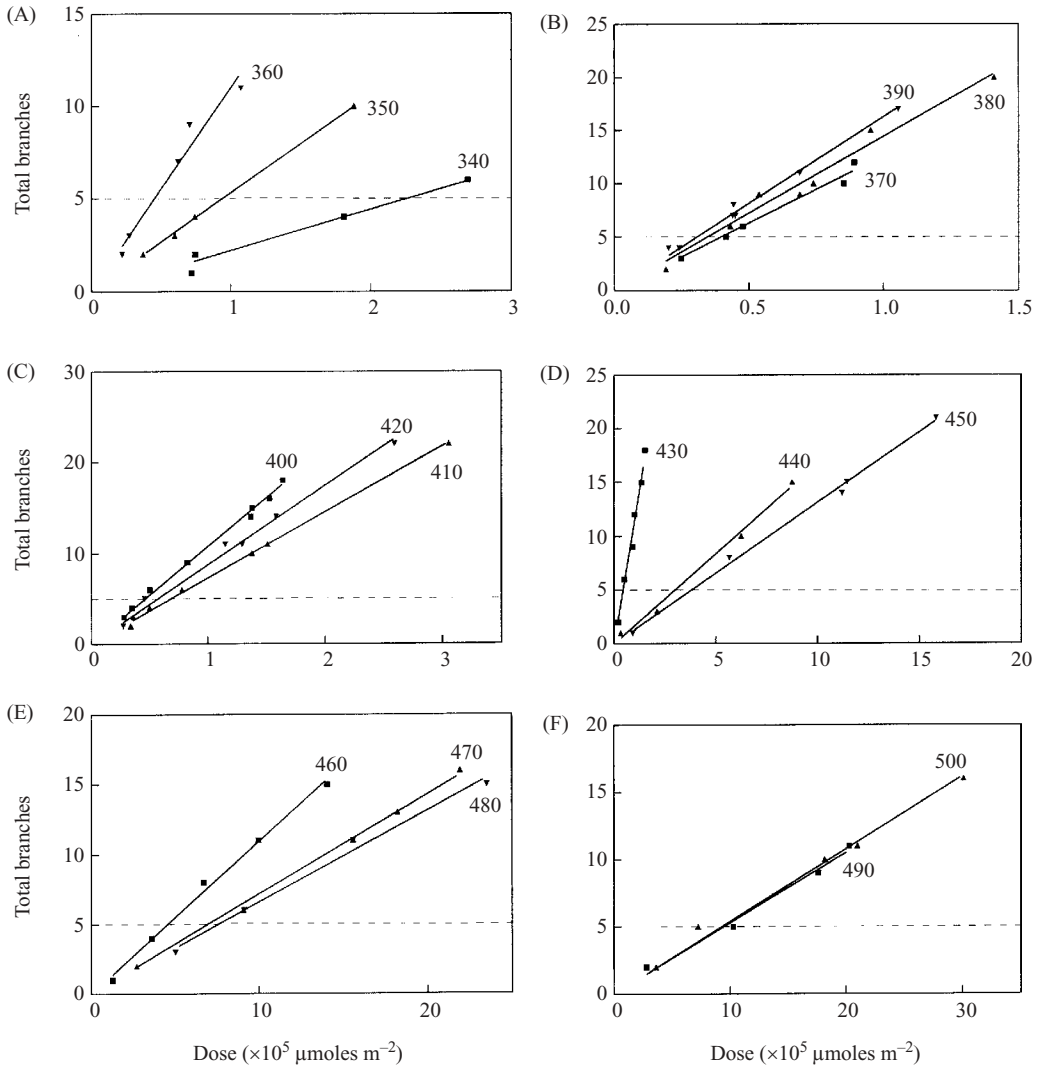

Fig. 1. The dose dependent response of hyphal branching of an arbuscular mycorrhizal fungus (*Gigaspora gigantea*) to monochromatic light between 340–500 nm at 10 nm increments. Between 4 to 9 different doses of light were used at each wavelength tested. Dashed line is the standard effect. (A) 340–360 nm; (B) 370–390 nm; (C) 400–420 nm; (D) 430–450 nm; (E) 460–480 nm; and (F) 490–500 nm.

3.8 μm s^{-1} m^{-2} showed two major peaks of branching stimulation, one at 390 nm and a second at 430 nm (data not shown). Long-term exposures at $\leqslant$340 nm inhibited germ tube growth.

Time course experiments indicated a linear response with respect to the number of hyphal branches *versus* exposure times between 4–24 h for most wavelengths tested, although at certain wavelengths the linearity only held up for 16 h (data not shown). To determine the action spectra for photoinduction of hyphal branches, dose response experiments were performed at 10 nm intervals between 340–700 nm (Figs 1–2). For the intensities used in these experiments, linear dose responses were observed for all the wavelengths tested (Figs 1–2) except for below 340 nm or above 670 nm. At $\leqslant$340 nm, long-term exposure actually inhibited hyphal growth with the germ tube tip frequently turning black. The induction of five hyphal branches was chosen as the standard effect, and the total μmoles m^{-2} of light necessary to induce the hyphae was determined for each wavelength tested. The standard effect was not achieved at 680–700 nm even after a 24 h exposure at high light intensity. The reciprocals of the dosage needed to produce the standard effect were plotted as relative quantum effectiveness *vs* wavelength to obtain the results shown in Fig. 3. This action spectrum showed that the major peak of activity was near 390 nm, with a shoulder between 360–370 nm. A second peak of activity was prominent near 430 nm.

Irradiation time and fluence rate response curves for filtered blue light

A time course (Fig. 4A) was initially done by exposing the first 8 mm of the germ tube to a high intensity blue light (145 μmoles s^{-1} m^{-2}). The number of branches induced was linear with respect to time up to 30 min. Micrographs showing the actual branching of germ tubes exposed for 10, 20, and 30 min are shown in Fig. 5. Fig. 5D shows that a major secondary hypha,

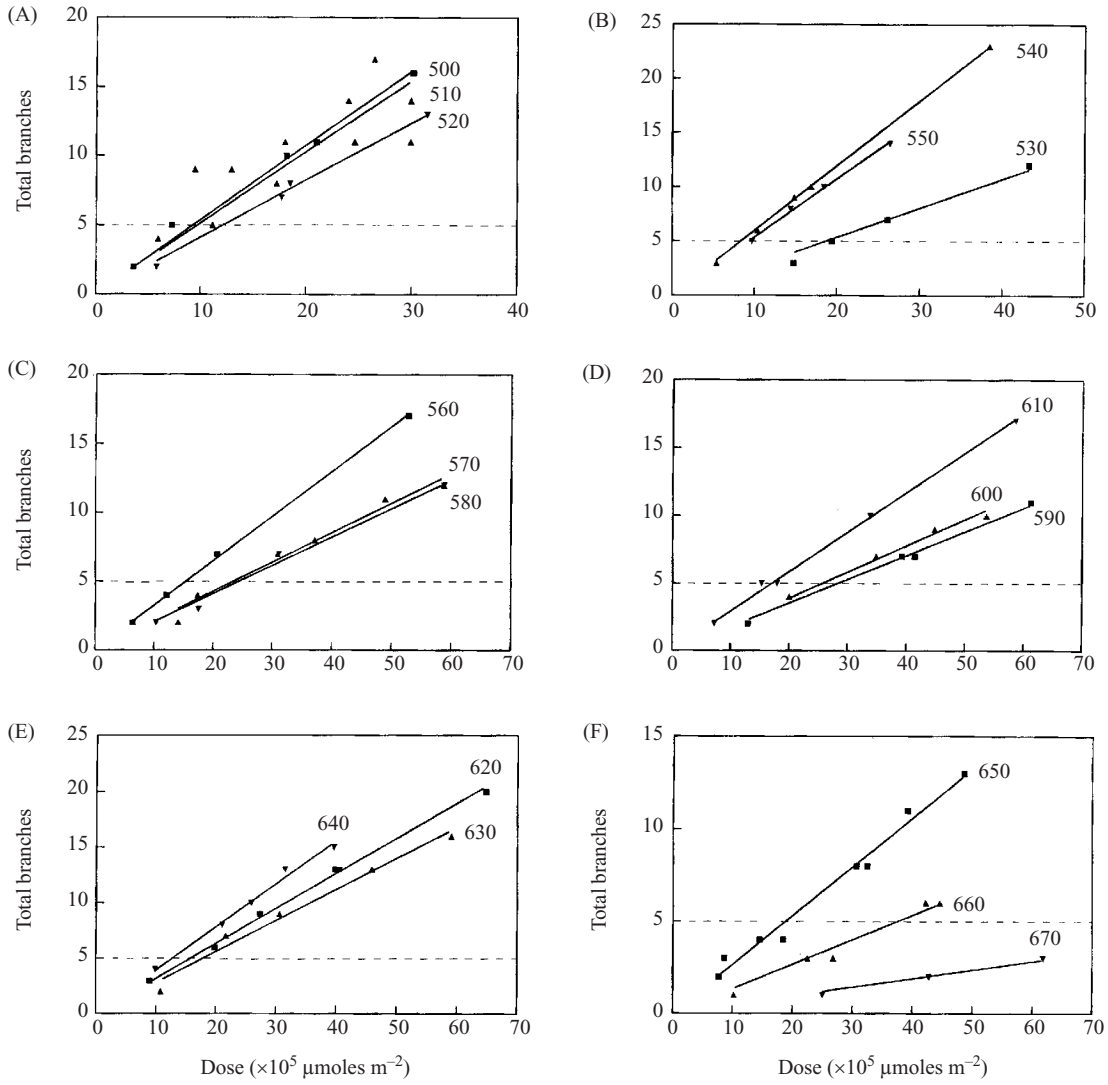

Fig. 2. The dose dependent response of hyphal branching of the an arbuscular mycorrhizal fungus (*Gigaspora gigantea*) to monochromatic light between 500–670 nm. 4–9 different doses of light were tested at each wavelength shown. Dashed line is the standard effect. (A) 500–520 nm; (B) 530–550 nm; (C) 560–580 nm; (D) 590–610 nm; (E) 620–640 nm; and (F) 650-670 nm. No branching was stimulated after 24 h of high-light intensity at 680–700 nm.

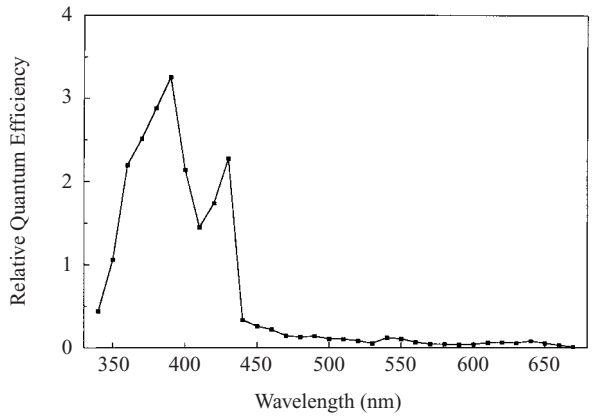

Fig. 3. The action spectrum for light-induced hyphal branching of the arbuscular mycorrhizal fungus, *Gigaspora gigantea*. The reciprocals of the values on the X-axis corresponding to five branches in Figs 1–2 were plotted as Relative Quantum Efficiency *versus* Wavelength.

which naturally curves, also responds to high intensity blue light. In fact, any growing hyphal tip of *Gigaspora gigantea* can be induced to branch with blue light.

The fluence rate response curve was linear up to at least 150 μmoles s^{-1} m^{-2} using a 30 min exposure with varying light intensity (Fig. 4B). Although the light intensity used here (25–150 μmoles s^{-1} m^{-2}) was much higher than that used to determine the action spectrum (0.5–3 μmoles s^{-1} m^{-2}), the total μmoles of light at 390 nm were very similar ($1.0–2.0 \times 10^5$ μmoles m^{-2} for Fig. 1B, and $1.0–2.0 \times 10^5$ μmoles m^{-2} after 20 min in Fig. 4A).

Exposure and hyphal branching sites along the axis of the germ-tube

For the subsequent experiments, only the primary germ-tube was used because its mode of growth was

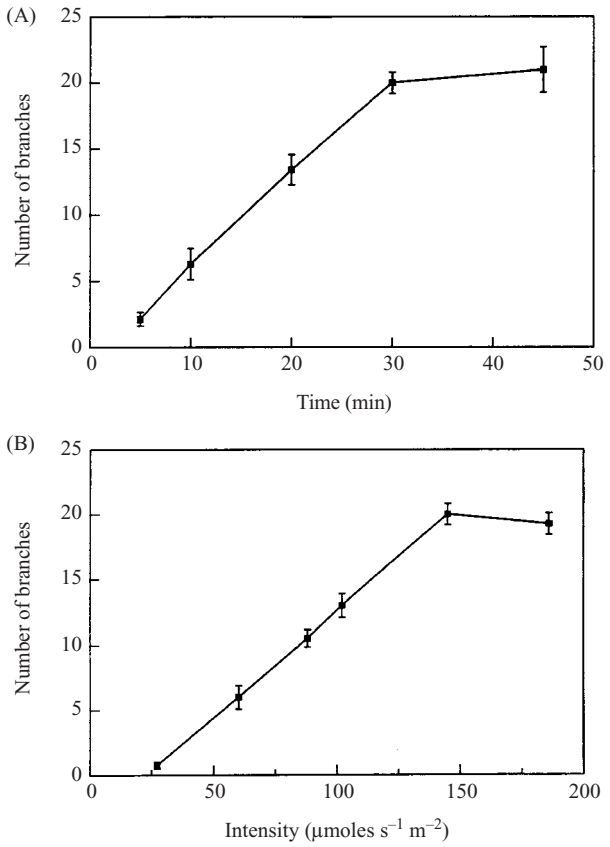

Fig. 4. Hyphal branching of the primary germ tube of *Gigaspora gigantea* induced by filtered blue light. (A) Time course for induced hyphal branches using an intensity of 145 μmoles s^{-1} m^{-2}. (B) Fluence rate response for hyphal branching. The exposure time was held constant at 30 min while the intensity was varied.

negatively geotropic and linear (straight up). Short-term exposure to high light intensity was necessary to minimize any change in the position of a tip during the exposure time. For the first experiment, 1 mm segments, working away from the germ-tube tip, were exposed but the number of induced branches was small and results were inconsistent (data not shown). The length of the apical tip exposed to light then was varied between 1–5 mm (Table 1). Maximum branching was induced when the apical 4 mm of the germ-tube was exposed. When 2–3 mm long segments were exposed at various distances from the tip (Table 2), the results confirmed that photoinduction of hyphal branches occurred anywhere along the axis of the germ-tube except in the first 2 mm of the tip.

A most interesting observation was made when segments 3 mm long were exposed at further distances from the tip. Fig. 6 showed that when a 3 mm segment (either 6–9 mm or 9–12 mm from the tip) was exposed to blue light, branching was induced only between the exposed area and the germ-tube tip, and not between the exposed site and the spore. Most significantly, a cluster of branches occurred in the exposed area but other distinct clusters of branches formed in areas not directly exposed to light. These results (Fig. 6) indicated that branching can occur at sites not directly exposed to light, and that branching clusters were usually spaced 3 mm or 6 mm apart between the exposed area and the germ-tube tip.

It could be argued that light only scatters upward in our system, so another experiment was conducted to investigate this possibility. The germ-tube was made into an U shape (Fig. 6C). Two days after germination, the Petri plate was turned 90 ° counterclockwise and incubated for 24 h, and then turned another 90 ° counterclockwise. After the last turn, the germ-tube was allowed to grow for 2 d more. A 3 mm segment was then exposed (Fig. 6C) and observed after 16 h growth in the dark with 2% CO_2. Branching was induced below the exposed site but, as before, the branches only occurred between the hyphal tip and the exposed site and not between the exposed site and the spore.

DISCUSSION

To date, three types of eukaryotic blue-light photoreceptors have been identified. Recently, a blue light activated adenylyl cyclase which mediates photoavoidance *in Euglena* was reported (Iseki *et al.* 2002). The phototropins are involved in various 'movement' type of responses of plants to blue light and this topic has recently been reviewed (Lin 2002). Chloroplast movement in relation to light intensity, hypocotyl phototropism, and stomatal opening are all mediated by phototropins (Lin 2002). Cryptochromes are more ubiquitous since they are found in plants, animals, and fungi (Horwitz & Berrocal 1997, Cashmore *et al.* 1999) and they mediate a variety of responses. Cryptochromes were historically defined by their action spectra with a typical broad band in the UV-A region and a second band in the blue region (Briggs & Huala 1999, Lin 2002).

Although little is known about light reception in AM fungi, the action spectrum reported here (Fig. 3 shows most efficient hyphal branching at 390 and 430 nm) was consistent with that of a cryptochrome and very similar to the action spectrum for light-induced conidiation of *Trichoderma* (Horwitz & Berrocal 1997). The action spectrum for conidiation had a major peak in the UV-A range (375 nm) and a second major peak in the blue light (440 nm) region. Action spectra were not reported for blue light-induced hyphal branching of the ascomycete *Neurospora genus* (Lauter *et al.* 1998), the basidomycete *Schizophyllum commune* (Raudaskoski & Viitanen 1982), nor of the oömycete (Grinberg & Heath 1997), so no comparison could be made with our results. The AM fungal blue and UV-A receptor appears to be a cryptochrome, and although there has been extensive work on non-AM fungal genetics (such as *Phycomyces* and *Neurospora*) and a large molecular genetic framework is available, only recently has a blue light photoreceptor been identified for *Neurospora* (He *et al.* 2002).

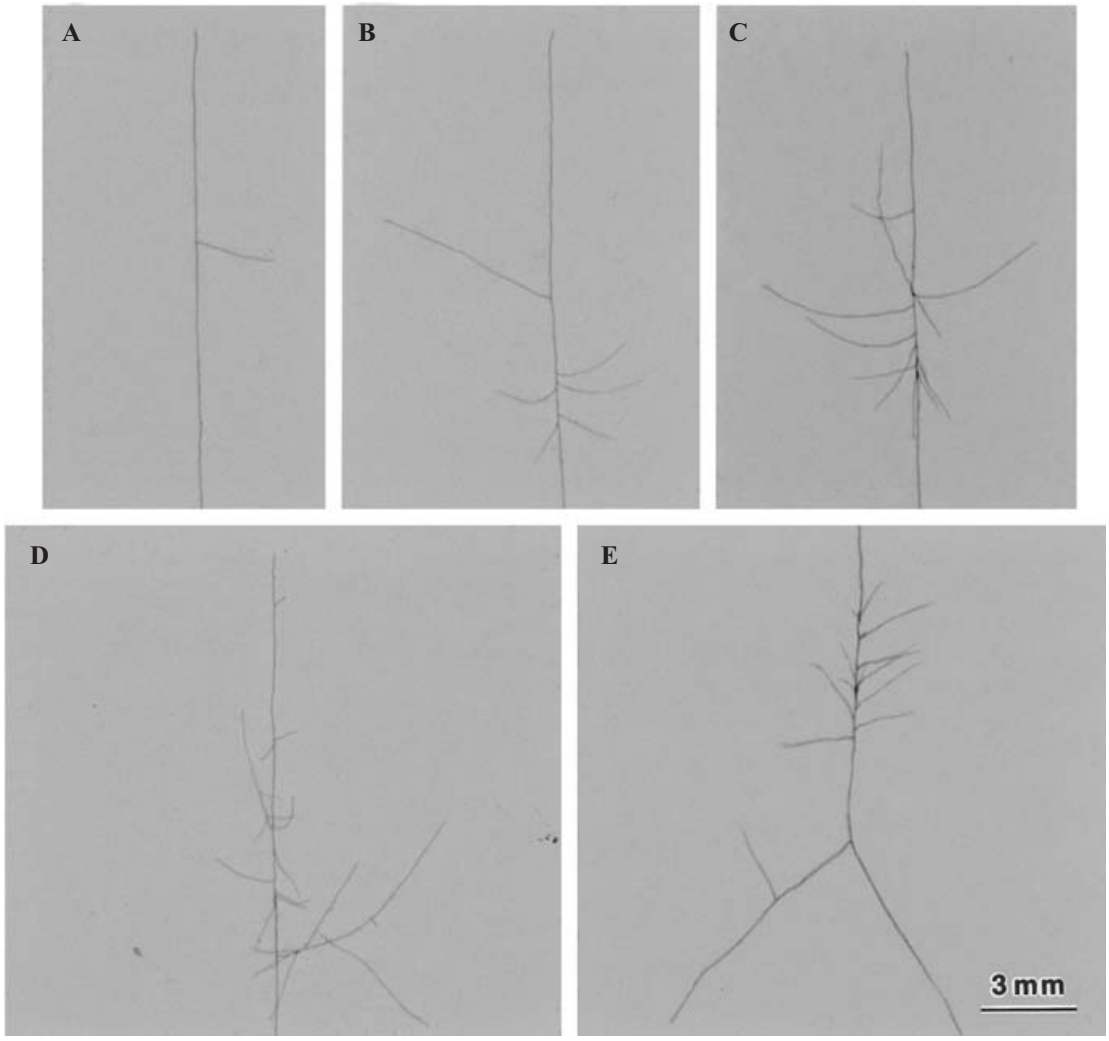

Fig. 5. Micrographs of blue light-induced hyphal branching of *Gigaspora gigantea* at high light intensity (145 μmoles s^{-1} m^{-2}). The first 8 mm of the germ tube was exposed for these experiments since this was the condition used to determine the action spectrum (Fig. 3) for light induced hyphal branching. (A) Dark control. (B) Branching of a primary germ tube after a 10 min exposure. (C) Primary germ tube branching after a 20 min exposure. (D) Primary germ tube branching after a 30 min exposure. (E) Branching of a main secondary hypha after 30 min.

A cryptochrome has also shown responsiveness to green light. The overexpression of a cryptochrome (CRY1, 75-kDa flavoprotein called cryptochrome 1) in transgenic tobacco plants (Lin *et al*. 1995) resulted in hypersensitivity to blue light, UV-A, and green light (490–570 nm). Likewise, the action spectrum reported here has a minor peak in the green light range (although not apparent in Fig. 3, it can be seen in the dose dependent response in Figs 2B–C). It may be that where the action spectra of cryptochromes exhibits a finer structure in responses to other wavelengths, many of the previous action spectra for cryptochrome responses (Horwitz & Berrocal 1997) were reported for a narrower range of wavelengths (typically 340–500 nm).

When small segments of the germ tube between 6–9 and 9–12 mm from a hyphal apex were exposed to blue light (Fig. 6), the branches induced often occurred in clusters in areas not directly exposed to light. In *Gelasinospora reticulispora*, light scattering outside of the microbeam exposure area during the photoinduction of perithecia could not be completely ruled out (Inoue & Furuya 1978). Perithecial formation did not occur outside of the exposed area if the exposed hyphae were septate and without protoplasm. However, light scattering by protoplasmic contents could not be ruled out. In our study, light-induced hyphal branching only occurred with an actively growing germ tube; however, protoplasmic light scattering could be ruled out since branching did not occur in both directions away from the exposed area. For some unknown reason, it could be argued that light only scattered upwards in our test system, but this was ruled out by Fig. 6C. In this case, branching occurred on the downside of the exposed area, but the branching still only occurred between the exposed area and the apical tip. The asymmetrical location of blue light-induced hyphal branches in relation to the exposure site was also reported for *Saprolegnia ferax* (Grinberg & Heath 1997). In this system, a blue light microbeam was focused on a growing hypha and branching normally occurred on

Table 1. Various lengths of growing tips of primary germ tubes of *Gigaspora gigantea* were exposed to high light intensity (150 μmoles s^{-1} m^{-2}) for 30 min to determine the location of the photoreceptors. After exposure, the plates were transferred to a dark CO_2 incubator for an additional 16 h before the branches were counted. The very apex of the tip is 0, so a 0–1 mm segment is the 1st mm of the tip. The numbers shown are average of seven determinations*.

	Length of exposed tip (mm)				
	0–1	0–2	0–3	0–4	0–5
Number of branches	0.29c	2.00c	10.14b	19.14a	18.14a

* Means of seven spores for each length of hyphal tip exposed to light. Numbers followed by the same letter are not significantly different (Tukey's test, $\alpha = 0.05$).

Table 2. Sequential segments of germ tubes of *Gigaspora gigantea* were exposed to high intensity light (150 μmoles s^{-1} m^{-2}) for 30 min. Starting at the hyphal tip and working back toward the spore, 2 mm wide segments and 3 mm wide segments were exposed. The numbers shown are the average of seven determinations*.

	Segment length (mm)				
	0–2	2–4	4–6	6–8	8–10
No. of branches for 2 mm wide segments	2.00b	18.00a	19.74a	18.67a	17.83a
	0–3	3–6	6–9	9–12	
No. of branches for 3 mm wide segments	10.14b	17.00a	21.00a	18.33a	

* Means of seven spores for each segment that was illuminated. Numbers in the same row followed by the same letter are not significantly different (Tukey's test, $\alpha = 0.05$).

the subapical side of the exposed site, the opposite of what we observed. Whether this has something to do with the germ tubes of *Gigaspora gigantea* being negatively geoptropic is not known.

Since branching can occur at sites not directly exposed to light, it is probable that a second messenger is involved. The second messenger appears to move only towards the growing tip. Alternatively, the photoreceptor itself may be mobile. AM fungi are multinucleate and lack cross-walls, and if fungal cryptochromes are either soluble (as was reported for *Neurospora*) and found in the cytoplasm or located in nuclei, the photoreceptors could move to the branching sites. If this is the case, a second messenger is still likely to be involved in the signal transduction process because another environmental cue, host root exudate compounds, can also induce hyphal branches (Giovannetti *et al.* 1993, Nagahashi & Douds 2000) of AM fungi. Signal transduction from the initial blue light exposure was reported for *Saprolegnia ferax*. When growing hyphae were irradiated with microbeams of blue light, an increase in cytoplasmic calcium was induced followed by early formation of hyphal branches (Grinberg & Heath 1997). Calcium ions are well known for their role as second messengers in eukaryotic cells.

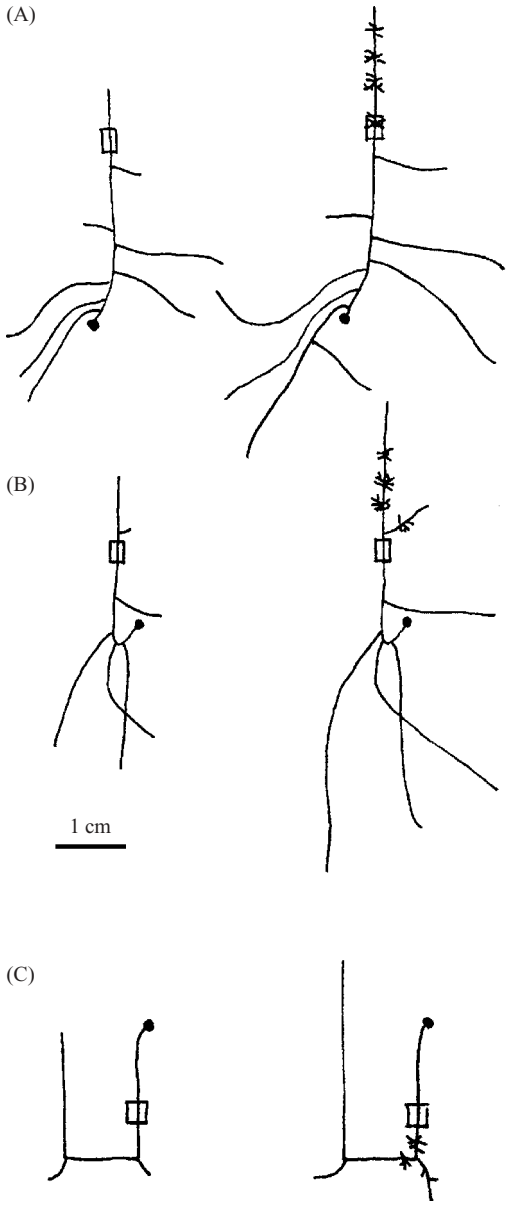

Fig. 6. Tracings of the blue light-induced hyphal branching of *Gigaspora gigantea*. Three different exposed segments of primary germ tubes are shown before (left side) and after illumination (right side). The germ tubes were exposed for 30 min at 145–150 μmoles s^{-1} m^{-2}. (A) A 3 mm long segment, 6–9 mm from the tip, was exposed. (B) A 3 mm long segment, 9–12 mm from the tip, was exposed. (C) The primary germ tube was made to make a U-shaped form by turning the Petri plate counterclockwise twice (see text for details). A 3 mm long segment, 33–36 mm from the tip, was then exposed. The main secondary hyphae were not traced in this figure. Immediately after light exposure, all Petri plates were transferred to an incubator with a 2% CO_2 atmosphere at 32 °C for 16 h, after which the the main hyphae and branches were traced right on the Petri dish.

The multiple branching clusters induced by blue light appeared to be periodic and were usually spaced 3–6 mm apart (Fig. 6). A similar periodicity in hyphal branching clusters of *G. gigantea* has also been shown when germinated spores were treated with concentrated

chemical compounds from host roots (Nagahashi & Douds 2000). Branching clusters induced by chemical compounds were usually 3, 6, and sometimes 9 mm apart. The chemical compounds from the host root exudate can stimulate hyphal branching anywhere along the growing axis of the germ tube, in the same manner that light can induce hyphal branches anywhere along the growing axis. Whether the chemoreceptor and photoreceptor are separate entities or whether there is only one type of receptor which responds to different environmental cues, remains to be determined. However, it would be reasonable to assume that a second messenger is activated or released by light or chemical signal during the signal transduction process.

The primary mechanism by which obligately symbiotic AM fungi find host roots in the soil is by responding to chemical signals from these roots. It is possible that the negatively geotropic germ tubes of *Gigaspora* spp. utilize the same mechanism, but in response to a different signal (i.e. light). Light-induced hyphal branching increases the probability of direct fungal contact with the roots of seeds that have germinated at or near the surface of the soil.

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